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13. ABSTRACT *(Maximum 200 words)*
Metabolic integration and mechanisms of nutritional exchange are important but poorly understood aspects of the physiology of symbioses between chemoautotrophic bacteria and marine invertebrates. The ability to generate organic nitrogen compounds from inorganic sources is a vital physiological capability involving coordination of host and symbiont metabolism. Glutamine synthetase (GS) catalyzes the first step in the incorporation of inorganic nitrogen, as ammonia, into organic nitrogen compounds. The relative importance of host and symbiont GS in nitrogen assimilation was determined in several representative mollusk and tubeworm symbioses. In most cases, the primary activity present in nitrogen-assimilating tissues is that of symbiont GS, a GSI similar to that of free-living Gram-negative bacteria. In contrast, in a coastal bivalve symbiosis, host GS predominated with symbiont GS expressed at low levels or inactivated. Further studies of symbiont GS expression were conducted to determine whether symbiont physiology is sensitive to alterations in energy supply to the host. Expression of symbiont GS, as well as symbiont densities and expression of Rubisco, was unchanged in deep-sea vent tubeworms subjected to short-term energy (sulfide) starvation but dramatically altered by chronic exposure to low sulfide. These findings are suggestive of short-term host buffering of symbiont nutritional supply as well as mechanisms of reducing symbiont populations in response to long-term stresses.

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Dr. Eric Eisenstadt
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Biomolecular Science and Technology Program
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800 North Quincy Street
Arlington, VA 2217-5660

7 August 1998

Dear Dr. Eisenstadt:

I am pleased to submit the Final Report on my ONR grant, for the project entitled "Molecular Characterization and Regulation of Ammonia Assimilation in Chemoautotrophic Prokaryote-Eukaryote Symbioses".

I certify that all of the requirements for the distribution of the report outlined in the letter by Dr. Harold E. Guard, Acting Director, have been completed. The report, 4 pages of text, includes Standard Form 298 as a cover, filled out as directed. Copies of the complete report have been sent to:

1. Defense Technical Information Center (DTIC): 2 copies + self addressed postcard.
2. Administrative Grants Officer (AGO): 1 copy.
3. Director, Naval Research Laboratory: 1 copy.
4. Program Officer at ONR: 2 copies with this cover letter.

I thank you and the Office of Naval Research for your interest and support of our research.

Sincerely,

Colleen M. Cavanaugh
Professor of Biology

cc: Defense Technical Information Center (DTIC)
Administrative Grants Officer (AGO)
Director, Naval Research Laboratory
Program Officer at ONR
Harvard University, Dept. of Organismic and Evolutionary Biology
Harvard University, Office of Sponsored Research

Encl./

FINAL REPORT

Grant#: N00014-97-1-0454

PRINCIPAL INVESTIGATOR: Colleen M. Cavanaugh

INSTITUTION: Harvard University

GRANT TITLE: Molecular characterization and regulation of ammonia assimilation in chemoautotrophic prokaryote-eukaryote symbioses

AWARD PERIOD: 1 March 1997 - 30 November 1997

OBJECTIVES: To determine the metabolic integration and mechanisms of nutritional exchange between host and symbiont in marine invertebrate-chemoautotroph symbioses by characterization of host and symbiont glutamine synthetase.

APPROACH: Host and symbiont glutamine synthetases (GS) of representative mollusk and tubeworm symbioses were investigated to determine their respective roles in nitrogen assimilation and gain insight into mechanisms of nutritional exchange.

ACCOMPLISHMENTS: Glutamine synthetase (GS) catalyzes the formation of glutamine from glutamate and ammonia and is the primary route by which nitrogen from inorganic sources is incorporated into amino acids by autotrophs. Characteristic differences in bacterial and eukaryote GS allowed symbiont and host GS to be distinguished in the intact association. All prokaryotes have a dodecameric form of GS termed GSI. A second form, GSII, is ubiquitous among eukaryotes and is also found in some root nodule bacteria and streptomyces which express both forms. Conservation of amino acid sequences between GSI and GSII is observed only in five regions associated with the catalytic site. GSI and GSII are otherwise distinct, differing in subunit size, isoelectric point, and sensitivity to thermal denaturation at 60°C. These differences facilitated the use of anion-exchange chromatography to separate and quantify GSI and GSII in symbiont-free and symbiont-containing tissues and development of specific antisera and probes.

GSI was the predominant form of GS in symbiont-containing tissues of the vent tubeworm *Riftia pachyptila*, clam *Calyptogena magnifica*, and mussel *Bathymodiolus thermophilus* but not in the coastal clam symbiosis *Solemya velum*. GSI eluted at high salt concentrations and exhibited the same elution profile as GSI from *Thiomicrospira* L-12, a free-living bacterium isolated from vents. Partially purified GSI was not inactivated by 60°C treatment and exhibited immunoreactivity to anti-*E. coli* GSI antiserum. GSI was not detected in symbiont-free tissues.

Host GS, GSII, was characterized in symbiont-free tissues of *R. pachyptila*, *C. magnifica*, *B. thermophilus*, and *S. velum*. Host GS eluted at low salt concentration and exhibited the same elution profile as GS from the vent worm *Alvinella pompejana* which does not contain endosymbionts. Partially purified host GS was not immunoreactive with anti-GSI antiserum and was inactivated by 60°C treatment. Host GS activity was generally very low to undetectable in symbiont-containing tissues.

The only GS that exhibited activity in symbiont-containing tissue of *S. velum* was identical to host GS in terms of thermal lability, immunoreactivity, and elution profile. Fractions eluting at the salt concentration of GSI from other species exhibited immunoreactivity to anti-GSI antiserum but no detectable activity, indicating that symbiont GSI is present but at low levels. Thus GS activity of symbiont-containing tissue appears to be entirely of host origin or may be due to a bacterial GSII, although Southern hybridizations with bacterial GSII probes have given negative results.

These findings indicate that the symbionts are capable of ammonia assimilation in *R. pachyptila, C. magnifica, B. thermophilus*. This differs from other prokaryote-eukaryote symbioses such as rhizobia and plants or cyanobacteria and *Azolla* in which symbiont GS activity is repressed and the host assimilates ammonia. It is also postulated that the host assimilates ammonia in marine symbioses between algae and invertebrates. Thus, unlike these other symbioses, the symbionts in chemoautotrophic symbioses function in assimilating ammonia into amino acids which could then be provided to the host by translocation or digestion of the symbionts. In *S. velum*, the symbionts may not assimilate appreciable ammonia or factors may be present that inactivate symbiont GS. GS-deficient symbionts are likely incapable of growth and as a result organic carbon from sulfide-based carbon dioxide fixation could be released to the host rather than incorporated into symbiont biomass. This is consistent with rapid organic carbon translocation observed in other species of *Solemya*.

To enable experimental manipulation of chemoautotrophic symbioses in the laboratory, flow-through aquaria were designed for maintenance of sulfide and oxygen at optimum levels. Studies of chemoautotrophic symbiosis have been limited by the inaccessibility of many species and difficulties maintaining these organisms in the laboratory. *S. velum* was selected as a model organism for developing laboratory maintenance protocols. *S. velum* kept in the flow-through aquarium were found to exhibit net uptake of carbon dioxide (autotrophy) for the duration of the time period tested (over 1 week). Symbiont densities and expression of enzymes of autotrophic carbon and nitrogen metabolism, Rubisco (the CO2 assimilation enzyme) and glutamine synthetase activity (total activity presumably host activity), were found to be stable in clams kept in this system. These findings indicates that this symbiosis retains autotrophic enzymes in the laboratory and that further experiments using *S. velum* as a model system are possible.

Studies investigating changes in symbiont GS expression in response to environment were conducted on the deep-sea hydrothermal vent tubeworm *Riftia pachyptila*. To determine whether symbiont GS expression is influenced by variation in autotrophy due to inevitable fluctuations in the vent water chemistry, *R. pachyptila* were collected from both active venting sites and peripheral locations where sulfide, the energy source for symbiont-based chemoautotrophy, is substantially reduced. Symbiont GS expression was undetectable in worms collected away from active venting, indicating that ammonia assimilation is reduced or absent in these worms. Similarly Rubisco was undetectable. Symbiont densities and ribosomal RNA levels were also substantially reduced. This is the first study to document changes in the physiology of the symbionts in response to changes in vent flow.

An opportunity presented itself to take part in an additional hydrothermal vent cruise to study living *R. pachyptila*. Using the same flow-through system that, for the first time, enabled *R. pachyptila* to be transported to and maintained at a land-based laboratory (see Science V279 p. 663), experiments were conducted in which sulfide levels were manipulated directly. Comparisons were made between worms kept with or without sulfide over a time-course of one week. During this period of sulfide starvation, symbiont GS expression, densities, volumes, and ribosomal RNA levels remained at basal levels indicating that the host will maintain (perhaps nutritionally)

its symbionts during short periods of starvation. This contrasts sharply with what was observed in worms from peripheral sites, which had been under conditions of sulfide restriction for an indeterminate, but likely greater than one week, period of time.

CONCLUSIONS: The glutamine synthetase of chemoautotrophic symbionts is a dodecameric GSI that has been characterized in other Gram-negative bacteria. In most chemoautotrophic symbioses, symbiont GS is the primary form present in symbiont-containing tissues, showing that the symbiont rather than host is responsible for the initial fixation of ammonia into amino acids. In contrast, in *S. velum*, symbiont GS, while present, has undetectable activity indicating that expression is very low or that the enzyme is inactivated by post-translational modification. In *S. velum*, novel mechanisms, differing from the more straightforward symbiont-mediated assimilation, may operate. Stable levels of GS expression (host in *S. velum*, symbiont in *R. pachyptila*) were observed in the laboratory. In *R. pachyptila* no changes in symbiont GS expression were observed in response to energy (sulfide) starvation for periods of up to one week. These results indicate that the symbiosis remains metabolically poised to assimilate inorganic nitrogen despite conditions that do not allow growth. GS expression of *R. pachyptila* symbionts was anticipated to be altered in the face of sulfide starvation. The results obtained are suggestive of buffering of the symbiont nutrient supply by the host. In contrast, field collected *R. pachyptila* from areas of very low sulfide availability can lose the bulk of their symbionts with a concomitant reduction in the capability to assimilate inorganic nitrogen and carbon. Under these conditions, which likely reflect prolonged sulfide starvation, symbiont populations appear to be down-regulated. Further studies are needed to investigate the mechanisms that allow symbiont populations to be maintained under short-term energy starvation or reduced under prolonged starvation.

SIGNIFICANCE: These studies represent a major accomplishment in understanding mutualistic interactions between prokaryotic symbionts and marine invertebrate hosts. In particular, key aspects of the nitrogen nutrition of these associations, which have hitherto been poorly characterized, have been revealed through studies of symbiont GS. The use of cellular and molecular biology approaches have allowed insights to be gained into symbiont metabolism while in the intact association rather than in culture as in other studies of marine symbiosis. Studies of GS expression in conjunction with investigations of symbiont densities and ribosomal RNA levels have provided a snapshot of the metabolic integration between host and symbiont. Such information is of general importance in understanding integration in other prokaryote-eukaryote mutualisms as well as organelles within eukaryotic cells. Investigations of the dynamics of interactions between marine bacteria and their hosts in mutualistic associations can potentially lead to insights into development of effective strategies for remediation of parasitic or nuisance associations that promote fouling on organisms and structures in the sea or result in human health threats when contaminating commercial fisheries.

AWARD INFORMATION: PI (C.M. Cavanaugh) awarded Phi Beta Kappa Teaching Prize, Harvard University, elected Fellow, American Association for the Advancement of Science and American Academy of Microbiology, American Society for Microbiology; Postdoctoral Fellow (R.W.Lee) appointed as Assistant Professor, Department of Zoology, Washington State University.

PUBLICATIONS AND ABSTRACTS (for total period of grant):

Lee, R.W., Robinson, J.J., Cavanaugh, C.M. 1998. Pathways of inorganic nitrogen assimilation and expression of host and symbiont glutamine synthetase in chemoautotrophic bacteria-marine invertebrate symbioses. (submitted).

Lee, R.W., Cavanaugh, C.M. 1998. Site-specific differences in molecular and cellular indices of symbiont autotrophy in the hydrothermal vent tubeworm symbiosis *Riftia pachyptila*. (in preparation).

Hektor, H.J.,, Lee, R.W., Robinson, H.J., Cavanaugh, C.M. 1998. Phylogenetic relations of three autotrophic marine symbionts based on their Rubisco sequences. (in preparation).

Lee, R.W., Cavanaugh, C.M. 1997. Symbiont glutamine synthetase in chemoautotrophic bacteria-invertebrate symbioses. Presented at International Symbiosis Congress, Woods Hole, MA.

Lee, R.W., Cavanaugh, C.M. 1997. Symbiont glutamine synthetase in chemoautotrophic bacteria-invertebrate symbioses. Presented at Boston Bacterial Meeting, Cambridge, MA.

Lee, R.W., Childress, J.J., Cavanaugh, C.M. 1997. Nitrogen assimilation by chemoautotrophic symbioses. Presented at First International Symposium on Deep-sea Hydrothermal Vent Biology, Funchal, Madeira, Portugal.

Lee, R.W., Cavanaugh, C.M. 1996. Pathways of inorganic nitrogen assimilation in marine invertebrate-chemoautotroph symbioses. Presented at Symbiosis 96 meeting, Bar Harbor, Maine.